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**14. ABSTRACT**

Recently, it has been recognized that a distinct mechanism of terminal proliferation arrest after chemotherapy involves the reactivation of senescence. However, whether this phenotype occurs in vivo is unclear, as is the biological impact of senescence induction. We have previously identified pathways and genes involved in human senescence that may serve as senescence markers, and have demonstrated that senescence occurs in prostate cancer cell lines after chemotherapy. In this proposal, we will: a) determine whether senescent tumor cells alter the proliferation and invasion of surrounding prostate cancer cells in vitro and in vivo, b) assess for and augment senescence in prostate cancer xenograft models and human tumors, and c) identify novel small molecules that induce senescence in prostate cancer cells. Both in vitro and in vivo approaches using human prostate cancer cells will be utilized to identify and determine the mechanisms underlying senescence. With this data, our understanding of cellular senescence will undergo a quantum leap and permit the translation of this entity both as a marker of response and for directing therapy..

**15. SUBJECT TERMS**

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## Introduction:

Senescence is an irreversible process that limits the lifespan of normal cells. It is believed to represent a tumor-suppression mechanism that is lost during neoplastic transformation. The induction of accelerated senescence, like other damage responses such as apoptosis, is a programmed response to a carcinogenic or biological insult involving multiple molecular pathways. It has recently been appreciated that senescence may also be a cytostatic response *reactivated* in tumor cells in response to chemotherapeutic agents. A limiting factor in identifying and therapeutically exploiting this phenotype has been the lack of molecular markers. In the attached manuscript we present evidence for a panel of senescence-specific molecular markers upregulated in both replicative and induced senescence. We also demonstrate that induction of a senescent phenotype in prostate cancer lines using doxorubicin inhibits growth of untreated cancer cells. It is our **hypothesis** that the therapeutic activity induced by chemotherapeutic agents is due, in part, to a senescence-like program of terminal growth arrest. Furthermore, this phenotype inhibits the proliferation of surrounding cells and its presence may predict tumor response to therapy.

## Body:

**Task 1**: **To determine whether senescent tumor cells alter the growth of surrounding prostate cancer cells *in vitro* and *in vivo***.

1. Co-culture and transwell experiments with ratios of senescent and proliferating cells; Generate senescent DU145 and LNCaP using DAC, doxorubicin and Docetaxel; proliferation and cell count; viablity (months 1-9)
2. Boyden chamber assays using ratios of senescent and proliferating DU145 and LNCaP cells (months 3-12)
3. *In vivo* studies using ratios of senescent and GFP-labeled non-senescent DU145 and LNCaP cells (10 animals per tx group; Total 50 for DU145 and 50 for LNCaP); GFP analysis cell count, BrdU proliferation, PI for viability, TUNEL/PARP for apoptosis. Statistical analyses (months 3-12)
4. If an effect on proliferation or invasion is seen then (months 6-24):
5. Repeat transwell and Boyden experiments with neutralizing antibodies to IGF receptors 1 and 2, (if stimulatory response) after western confirmation.
6. Repeat transwell and coculture experiments with neutralizing antibodies to IGFBP3 and 5(if inhibitory response)
7. Selective downregulation of putative effectors in senescent cells using siRNA

**Completed**. This paper entitled "**Drug-Induced Senescence Bystander Proliferation In Prostate Cancer Cells *In Vitro* and *In Vivo*"** is attached (appendix 1) and has been published. The data and methods (Subtasks 1-6) are included within this manuscript. Research on subtask 7 has not been approached since inhibition of IGF2 prevented the modest bystander effect seen with the senescence phenotype.

Senescence is a distinct cellular response induced by DNA damaging agents and other sublethal stressors and may provide novel benefits in cancer therapy. However, in an aging model senescent fibroblasts were found to stimulate the proliferation of co-cultured cells. To address whether senescence induction in cancer cells using chemotherapy induces similar effects, we used GFP-labeled prostate cancer cell lines and monitored their proliferation in the presence of proliferating or doxorubicin-induced senescent cancer cells *in vitro* and *in vivo*. Here we show that the presence of senescent cancer cells increased the proliferation of co-cultured cells *in vitro* through

paracrine signaling factors, but this proliferative effect was less than that seen with senescent fibroblasts. *In vivo,* senescent cancer cells failed to increase the establishment, growth or proliferation of LNCaP and DU145 xenografts in nude mice. Senescent cells persisted as long as 5 weeks in tumors. Our results demonstrate that while drug-induced senescent cancer cells stimulate the proliferation of bystander cells *in vitro*, this does not significantly alter the growth of tumors *in vivo*. Coupled with clinical observations, these data suggest that the proliferative effects of senescent cancer cells are negligible and support the further development of senescence induction as therapy.

**Task 2**: **To assess for and augment senescence in prostate cancer xenografts and human tumor tissues.**

1. Generate Du145 and LNCaP xenografts in nude mice (months 6-24)
2. Treat with Docetaxel or doxorubicin and harvest at 3 intervals (3 intervals X 10treated/10control per xenograft line = total 60 for DU145 and 60 for LNCaP). GFP analysis cell count, BrdU proliferation, PI for viability, TUNEL/PARP for apoptosis (months 12-30)
3. QPCR and immunohistochemistry for senescence markers (months 12-36)
4. Analysis of human neoadjuvant tissues (10 treated/10 untreated per trial X 2). QPCR and immunohistochemistry for senescence markers (months 24-36)
5. Statistical analyses and correlation with proliferation

Current work on Task 2:

Subtasks 1 and 2: We have set up these experiments and completed harvesting these mouse tumors. Analyses are currently in progress. Nude mice (10+ group) containing DU145 or LNCaP xenografts were treated with Docetaxol(10 mg/kg), Doxorubicin (5mg/kg), or vehicle on Days 0, 2, and 4 (3 experiments). BrdU pellets were implanted on the last treatment day. Animals were sacrificed 5 days after last dose. Tumors harvested for RNA, protein, sectioned for BrDU staining and SA B-gal. Treatment results are displayed in Table 1.

| | **DU145** | **LNCaP** |
|---|---|---|
| **Docetaxol** | TV= -23%<br>BrDU= -17%* | TV= -52%<br>BrDU= -31% |
| **Doxorubicin** | TV= -33%<br>BrDU= -35% | TV= -39%<br>BrDU= -33%<br>SA Bgal + |

**Table 1: Tumor volume (TV) and Proliferation (BrDU) in Prostate Cancer Xenografts Harvested after 4 days.**

Subtask 3: SA-Bgal expression was only found with doxorubicin treatment. We additionally ran RNA for 9 senescence marker genes (Fu et al., Neoplasia, 2007) with the following induction results: Du145/Doxorubicin (1/9), LNCaP/Doxorubicin (8/9), Du145/Docetaxol (4/9), LNCaP/Docetaxol (1/9). Thus, we are able to induce a growth inhibition, and in LNCaP a senescence phenotype is found after treatment with doxorubicin, and not docetaxol. Further work with novel agents that induce senescence is detailed in Task 3.

Subtask 5: No statistical correlation with proliferation was noted in the xenograft samples when proliferation was correlated with BrDU uptake.

Subtask 4: We will be investigating additional markers of senescence focusing on senescent-associated histone acetylation changes to determine if any are sensitive enough to reliably detect senescence in human tumors.

**Task 3**: **To screen for small molecules capable of inducing senescence.**

1. Generate senescence reporter construct using CSPG2 and stably transfect prostate cancer cell lines DU145 and immortalized human prostate epithelial cell line HPV16E7. Select and test reporter. (months 1-6)
2. Optimization of detection conditions (months 6-12)
3. Screen 500 compounds with DU145 to gauge appropriate concentration
4. Screen full 16,000 compound library (months 12-18)
5. Secondary analyses of 25 most active compounds in other prostate cancer cells lines including QPCR for senescence markers, morphology, cell cycle arrest and SA B galactosidase staining. (months 18-30)

Current work on Task 3:

Subtask 1: We generated a reporter construct for *cspg2* containing luciferase and transiently transfected it into the Du145 cell line. Unfortunately, when pooled transfectants were exposed to senescence-inducing doses of doxorubicin (25uM), we were unable to generate a reliable readout for senescence due to low expression levels. The failure of this aim, lead to the idea that simply looking at cell number would allow an initial screen and this could be combined with SA-B-gal expression and morphology to screen for senescence induction.

Subtask 2-5: **A high-throughput senescence screen identifies novel compounds.** We developed a high-throughput, phenotypic screen to identify compounds in chemical libraries that induce the characteristics of cellular senescence in prostate cancer cells. DU145 was chosen as a model for advanced prostate cancer based on its androgen-independent growth, mutant p53 status, and ability to develop a strong senescent phenotype. The screen is based on the pairing of two compatible staining techniques; one that identifies growth inhibition, and the other SA-β-gal activity (Fig. 1A). The fluorescence of the DNA binding agent Hoechst 33342 was measured to determine cell number after compound exposure for 3 days. In validation studies, the average fluorescence of wells with proliferating cells versus cells induced to senescence with 25nM doxorubicin demonstrated an acceptable Z'-factor of 0.53. This screening-window coefficient indicates a high signal-to-noise and signal-to-background ratio. As this measurement does not differentiate between the induction of senescence or apoptosis, wells with low fluorescence were subsequently visually assessed for SA-*β*-gal staining and senescent morphology.

To identify senescence-inducing compounds, we screened a pilot library of 4160 known bioactive compounds and natural products (KBA) containing structurally diverse characterized compounds, drugs, pollutants and naturally occurring extracts. Using a dose of 10μM in a 96-well format, Hoechst 33342 staining resulted in 625 initial hits (Fig 1B). Compounds with fluorescence >1 standard deviation less than the average of "per plate" data were selected. Wells containing both SA-*β*-gal staining and a senescent morphology (51 compounds) were then assessed for their ability to induce a persistent growth arrest. In triplicate wells, cells were replated and exposed to each of the 51 compounds for 3 days, then allowed to recover following drug removal for an additional 3 days. Cells treated with 9 of the 51 compounds maintained their arrested growth state after removal of the drug (indicated by unchanged Hoechst 33342 intensity; data not shown).

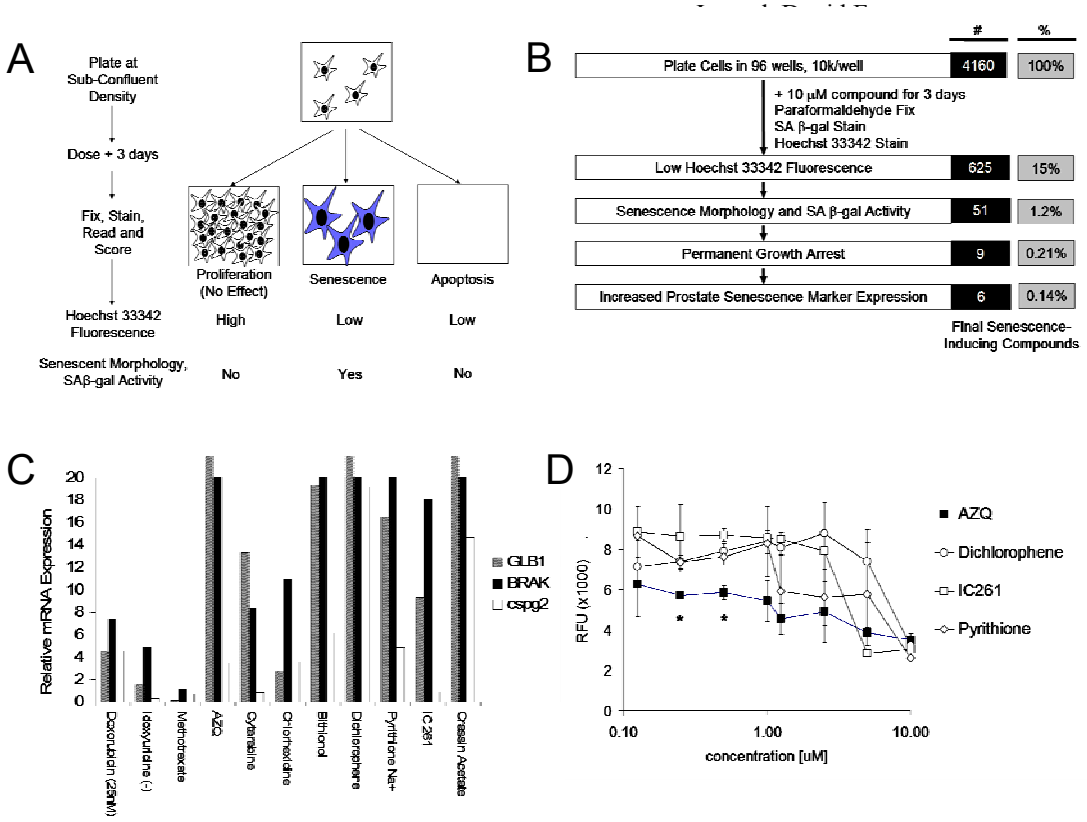


**Fig. 1. Screen for senescence-inducing compounds.** *A.* Du145 prostate cancer cells were plated on 96 well plates and utilizing robotic high-throughput screens, compounds from a library are plated. After 3 days, proliferation is determined by fluorescence after staining with Hoechst 33342. Low signal wells, indicating either senescence or apoptosis, were then visually examined for the presence of SA-β-gal staining and a senescent (enlarged, flattened) morphology. *B.* Results of the screen on a 4160 compound known bioactive compounds and natural products (KBA) library. Secondary tests included permanent growth arrest, and the induction of other senescent markers. *C.* Expression of senescence marker genes *GLB1, BRAK* and *cspg2* in DU145 cells treated with candidate or control compounds measured by qPCR and normalized to 18S expression. Doxorubicin (25nM) was utilized as a positive control [fu 2006], and one of several quiescence-inducing compounds (idoxyuridine shown) represents a negative control. Data is shown from one experiment performed in duplicate. *D.* AZQ inhibits Du145 cell growth at lower concentrations than other identified compounds. Hoechst 33342 fluorescence was measured after 3 days in wells after treatment with decreasing compound concentrations. Data showing chlorhexidine, bithionol, cytarabine and crassin acetate effectively inhibited proliferation only at doses higher than 1μM are are not shown for sake of clarity. These data represent the results of two independent experiments performed in triplicate. Error bars represent one standard deviation.

These 9 compounds were then tested to determine if they induce the expression of the previously identified senescence marker genes *Glb1, Brak* and *Cspg2*. After a 3 day compound exposure, qPCR was performed on RNA extracted from Du145 prostate cancer cells. Robust induction of all markers was demonstrated with 6 compounds (Fig. 3C) when compared to several quiescence-inducing controls (idoxyuridine shown). This experiment was reproduced using the hormone-dependent LNCaP prostate cancer cell line, confirming robust induction of all senescence marker genes with 6 compounds. In sum, this screen has identified compounds (Table 1), out of an original 4160, based on multiple previously established senescence criteria. These compounds are mechanistically diverse, and several had previously been identified as demonstrating growth inhibitory activity in cancer cells.

**Table 1: Senescence-inducing agents identified by screening**

| Compound | PubChem ID | PubChem: Anti-Cancer Activity | | Reported Mechanism of Action |
|---|---|---|---|---|
| | | *in vitro* | *in vivo* | |
| AZQ | 42616 | + | - | DNA damaging / ROS |
| Cytarabine | 114682 | N/A | N/A | Inhibit DNA Synthesis |
| Bithionol | 2406 | + | - | ? |
| Pyrithione | 1570 | + | N/A | Zinc Ionophore |
| IC261 | 5288600 | N/A | N/A | CK1-γ and -ε inhibitor+ |
| Dichlorophene | 3037 | + | - | ? |

**The Compound AZQ Induces A Potent Senescence Growth Arrest *In Vitro* and *In Vivo*.** The relative potency of the identified compounds to inhibit cellular proliferation was tested. In 96-well plates, DU145 cells were treated with a range of compound concentrations (0.1-10uM) and the average well fluorescence measured after fixing the cells and staining them with Hoechst 33342 (Fig 1D). AZQ inhibited proliferation to a greater extent at sub-µM concentrations when compared to other identified compounds rendering it the most potent of these agents. Structurally, AZQ is a rationally-designed, lipophillic, DNA-alkylating quinone.

To demonstrate these results were not cell line specific, other prostate cancer cell lines were treated with AZQ and longer-term and complete growth inhibition was shown after drug removal (Figure 2A; p=0.01).

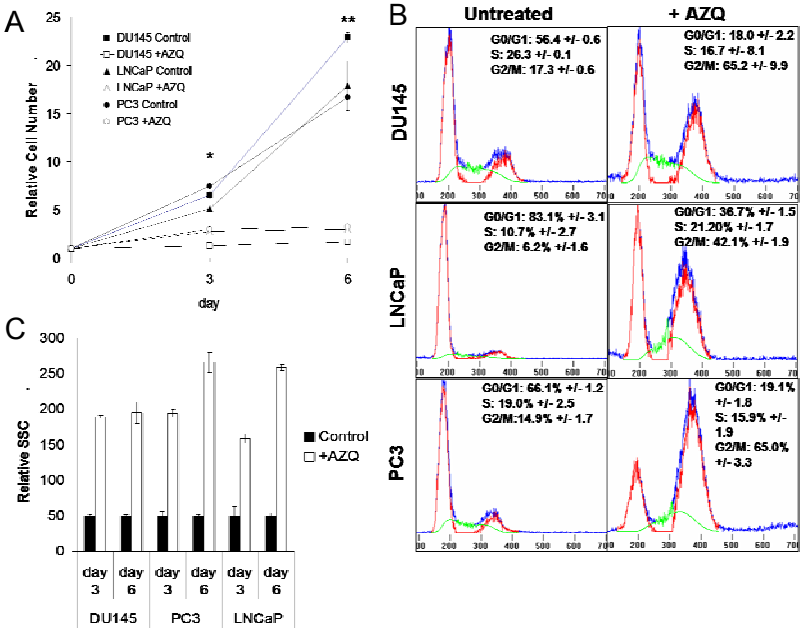


**Fig. 2. AZQ induces a terminal, senescence growth arrest in DU145, LNCaP and PC3 cells.** *A.* In multi-well plates, prostate cancer cell lines were dosed once with 250nM AZQ or vehicle and cell number determined by a cell sorter after staining with Hoechst 33342. Results are shown after 3 or 6 days in culture and normalized to day 0 data (*: p< 0.02. **: p<0.01). *B.* Treated and untreated cell lines were PI stained and subject to cell-cycle analysis after 3 days -/+ AZQ. Each panel represents three experimental replicates. The average values are shown in each respective panel, -/+ standard error. The differences in G0/G1 and G2/M fractions between cells -/+ AZQ is significant (p< 0.0001). *C.* Cell sorter analysis of cellular size and complexity (SSC). In samples treated with AZQ or vehicle and analyzed at 3 days relative units of SSC are shown (compared to control). Error bars represent standard error (A, C). (*: p< 0.001) These data represent the results of two independent experiments.

Analysis of DNA content in cell lines at the 3 day timepoint shows that AZQ-treated cells accumulate in G2/M and are significantly different than untreated cells (p<0.0001; Fig 2B). The broad distribution of this peak suggests the possibility that this population may include cells arrested at late S phase checkpoints as well. A second characteristic of senescence, increased cellular complexity and size, was measured by flow cytometry using side-scatter(SSC)(10). SSC in viable AZQ-treated cells was increased in all cell lines at both 3 and 6 days (p<0.001; Fig 2C). Viability is another feature of senescent cells. PI

exclusion demonstrates all treated cell lines maintain an average of 71% +/- 4% viable cells after exposure to AZQ at both day 3 and day 6 timepoints when compared to untreated cells ($p < 0.03$; Fig 3A). Western analysis of protein lysates from AZQ treated cells were analyzed to evaluate apoptosis. Both proliferating and senescent cell lines maintain similar amounts of full length PARP without any detectable cleavage products that would be indicative of apoptosis(33) (Fig 3B). Given this and the cell cycle analysis data, the response of these cells to AZQ is largely non-cytotoxic.

Prostate cancer cell lines were then stained for SAB-gal activity, a marker of senescence [ref] and staining graded from 0 (no staining) to 3 (intense, complete staining). At 3 days after treatment, increased SAB-gal activity was demonstrated in treated cell lines ( Fig. 3C).

Finally, we investigated whether AZQ induces a senescent phenotype *in vivo*. Previous studies had demonstrated in other tumor types an *in vivo* cystostatic response (25). As a model system, we generated DU145 xenografts roughly 1cm in size [ref] and treated them with a single intraperitoneal injection of 4 mg AZQ/kg body weight or vehicle (Fig 4). No toxicity was noted in the acute setting. Similar to *in vitro* results, increased SAβ-gal activity was observed in DU145 xenograft tumors of mice that were administered AZQ. By contrast, increased SAβ-gal activity was not observed in tumors from control mice injected with PBS vehicle (n=3). Apoptosis induction in these tumors, assessed using antibodies that specifically recognize cleaved PARP(33), showed minimal apoptosis in all tumors independent of AZQ treatment, suggesting that these molecular changes are not associated with apoptosis (data not shown). These results demonstrate the ability of AZQ to be effectively delivered *in vivo* and to induce SAβ-gal activity in DU145 prostate tumor xenografts.

In sum, AZQ to induce a phenotype consistent with senescence growth arrest. These data also validate the ability of our high-throughput screen to identify senescence-inducing compounds.

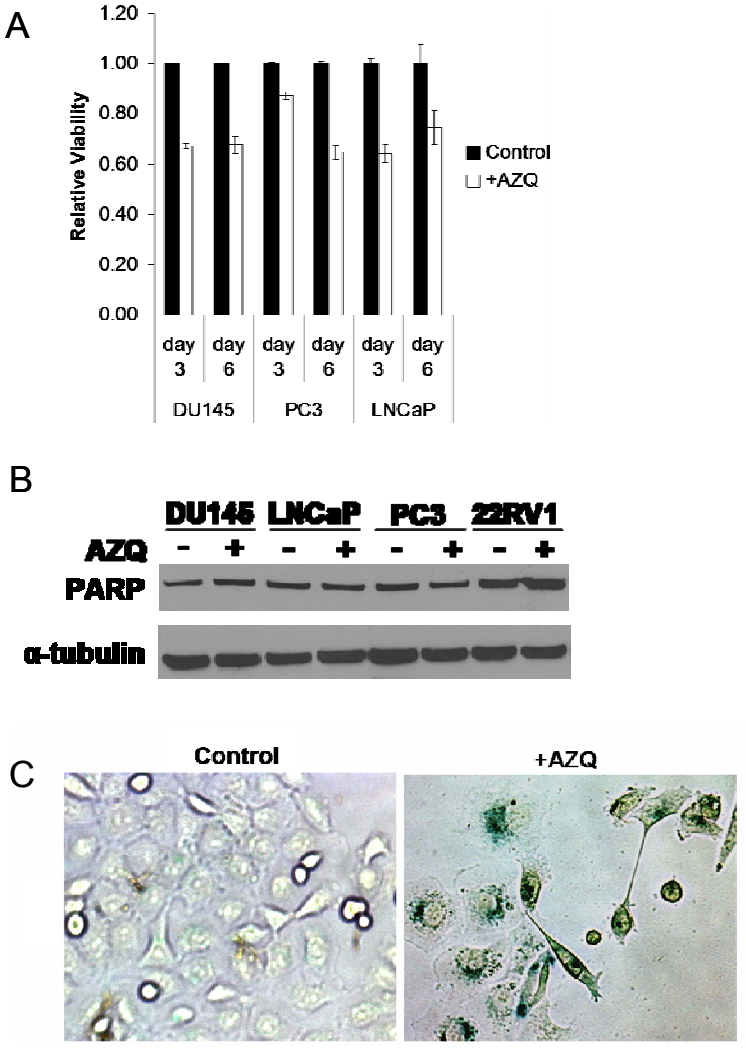


**Figure 3:** Exposure to senescence-inducing AZQ maintains viability. A. Viability of cells cultured -/+ AZQ, as measured by PI exclusion and cell size (forward scatter), normalized to data from untreated cell samples. (*: $p < 0.03$). Error bars represent standard error. B. Immunoblot analysis of full length PARP and a-tubulin expression in whole lysates of cells -/+ 250nM AZQ for 72 hr. These results are representative of three independent experiments. C. Detection of SAB-gal activity in whole DU145 cells cultured *in vitro* -/+ AZQ. Original magnification: 400x.

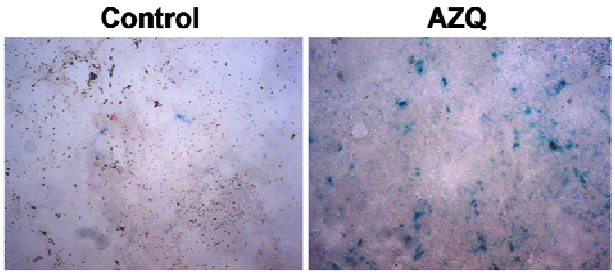


**Figure 4:** Bright field microscopy of SAB-gal activity in DU145 cells -/+ AZQ *in vitro*. These results are representative of 4 independent experiments.

A manuscript is currently in submission regarding these experiments and our identification of a novel robust senescence inducing agent AZQ. We have done additional experiments and find it to be and excellent cytostatic drug both in vivo and in vitro. Additional screening of a 16,000 compound library is ongoing.

## Key Research Accomplishments:

- Senescence induces a bystander effect in vitro, but not in vivo.
- *In vitro* senescence is mediated, in part, by the IGF axis
- A novel, whole-cell senescence screen identifies novel agents that induce senescence robustly.
- Senescence is able to be induced in xenografts in vivo with specific agents.

## Reportable outcomes:

1. September 5-8, 2007. IMPACT Department of Defense Prostate Cancer Meeting, Invited speaker "Senescence as therapy for prostate cancer", Atlanta, GA.
2. Ewald JA, Desotelle JA, Almassi N and **Jarrard DF**. Drug-induced Senescent Prostate Cancer Induces Proliferation *In Vitro* but Not *In Vivo.* (*British Journal of Cancer*)
3. Ewald JA, Desotelle JA, Laurila T, Almassi N and **Jarrard DF**. A Novel High-Throughput Screen Identifies Potent Senescence-Inducing Activity of Diaziquone (AZQ) (in review)

## Conclusions:

We conclude that while drug-induced senescent cells stimulate the proliferation of surrounding cancer cells *in vitro*, this does not significantly affect the long term growth of bystander cells that might escape senescence induction. These data support further development of senescence-induction strategies for cancer treatment. Furthermore, the above results validate the ability of this high-throughput assay to identify senescence induction. It provides a tool to develop novel senescence-inducing compounds for prostate cancer therapy, as well as providing further insight into mechanisms of senescence induction.